UNIVERSITÀ
DEGLI STUDI DI MILANO-BICOCCA

PhD Course in Translational and Molecular Medicine – DIMET XXXVIII cycle, a.y. 2022/2023

Scholarships

N. 2 linked to research projects:

1) "Deciphering the tumor-immune microenvironment and reprogramming tumor-infiltrating lymphocytes for glioblastoma T cell therapy
2) "Protein misfolding in neurodegenerative diseases: diagnostic applications and basic research"

Company: Fondazione IRCCS Istituto Neurologico Carlo Besta

Abstract:

Project 1
Glioblastoma (GBM) is a devastating brain cancer with median survival after recurrence of only 9 months, and no effective standard of care that can reverse this dismal prognosis.
Accumulating evidence suggests that GBM is not immunologically inert but susceptible to immune recognition, providing a glimmer of hope for the development of effective immunotherapy approaches. TILs can recognize and eliminate cancer cells but can become dysfunctional because of the immunosuppressive signals they receive within the tumor microenvironment (TME). Our preliminary results demonstrate that tumor-reactive TILs can be isolated from GBM, and successfully expanded in vitro in ~70% of cases. The expanded TILs show specific reactivity against autologous tumor cells, supporting the notion that their dysfunctional state is "reprogrammable" and providing a strong rationale for TIL immunotherapy in GBM. For the remaining cases, tumor-reactive TILs fail to expand, suggesting a terminal dysfunctional state.
We hypothesize that these two different dysfunctional states, reprogrammable vs. terminal, are imprinted into different transcriptional and epigenetic programs and driven by the immunomodulatory action of specific myeloid subsets in the TME.
The project aims to:
1) Characterize the immunophenotype and expansion ability of tumor-reactive TILs;
2) Reconstruct the transcriptomic and epigenetic programs underlying TIL dysfunction and reprogrammability;
3) Identify TME signals and myeloid subclasses implicated in TIL dysfunction; 4) Identify accessible biomarkers to predict TIL expansion and tumor progression.
The results and insights generated will pave the way to the future application of TIL therapy to ultimately bring clinical benefit to GBM patients, addressing one of the most pressing needs in oncology.

Project 2
Many neurodegenerative diseases are caused by the conformational conversion (misfolding) of specific proteins which aggregate and accumulate in the central nervous system (CNS). These proteins are considered disease-specific biomarkers and their identification in the CNS collected post-mortem is necessary to make a definitive diagnosis of the disease. Thanks to the development of innovative molecular biology techniques named Real-Time Quaking-Induced Conversion (RT-QuIC) and Protein Misfolding Cyclic Amplification (PMCA) it has been demonstrated that traces of these misfolded proteins

are present in peripheral tissues of patients, even at early disease stages. This project aims to analyze with standard and ultrasensitive techniques the cerebrospinal fluid, skin, urine, blood and olfactory mucosa collected from patients with different neurodegenerative diseases. The reaction products obtained after RT-QuIC or PMCA analysis will be subjected to biochemical and morphological (Transmission Electron Microscopy) analyses and inoculated in animal models (intracerebrally) or cell models to study their inflammatory and pathological properties. The results of this project will consent to determine if the peripheral distribution of disease-specific biomarkers changes at different stages of the disease and whether through the analysis of the RT-QuIC and PMCA reaction products it would be possible to stratify patients in the early disease stage.

N. 2 linked to research projects:

1) *"Finding key players in leukemia-stroma crosstalk as targets for new treatments in childhood Acute Lymphoblastic Leukemia"*
2) *"Characterization of the medullary niche of acute myeloid Leukemia: identification of new therapeutic targets"*

Company: FONDAZIONE M. TETTAMANTI M. DE MARCHI ONLUS

Abstract:

Project 1
Multidrug high doses chemotherapy against leukemic cells led the five-year event free survival rate of B-Cell Precursor Acute Lymphoblastic Leukemia (BCP-ALL) increase to more than 85% for children and approximately 40% for adults. Treatment failure might be due to the protective role of the altered leukemic bone marrow (BM) microenvironment, a sanctuary in which stromal cells dialogue with neoplastic B cells through soluble factors, extracellular vesicles (EVs) and metabolite exchanges. It has been demonstrated that the altered leukemic microenvironment plays a crucial role in sustaining, promoting leukemia and protecting malignant cells from conventional drugs. Thus, the identification of the molecules involved in the leukemia-stroma cross-talk would be fundamental for the establishment of a niche-targeted therapeutic strategy that could be administered in combination with conventional drugs. The general aim of the project will be to identify stroma-derived signals driving the maintenance and the evolution of leukemia in order to discover new molecular mechanisms that might be the targets for therapeutic intervention.

Project 2
Acute myeloid leukemia (AML) is a disease of the bone marrow, a disorder of hematopoietic stem cells due to genetic alterations in blood cell precursors resulting in overproduction of neoplastic clonal myeloid stem cells. Prognosis of patients affected by AML has improved over the last decades, with survival rates of patients reaching 40% in adults and 70% in children. Hematopoietic stem cells transplantation (HSCT) is the most effective option for preventing leukemia recurrence even if relapse after HSCT remains the main cause of treatment failure. Innovative strategies need to be implemented to improve the prognosis of patients. The bone marrow microenvironment (BMME)plays a critical role in the development, progression, and relapse of AML. It is now well-known that Leukemia/BMME interaction contributes to chemotherapy resistance and disease relapse. An important player in the BMME is represented by mesenchymal stromal cells, which are characterized by the ability of modulating their immunophenotype, secretory, metabolic and migratory properties depending on microenvironment conditions. Therefore, we aim to perform a deep characterization of AML BMME to identify new specific targets. In particular, specific antigens/soluble factors expression can be exploited for preferentially directing chimeric antigen receptor (CAR) T-cell therapyto the BM and increasing their local persistence, enhancing CART-cells potency in the cradle of leukemic stem cells. For instance, chemokine-mediated trafficking can be leveraged to enhance CART-cells activity. Chemokines and their receptors are crucial for the migration and homing of lymphocytes and play a critical role in the development and homeostasis of the immune system. Leukemia utilizes mechanisms such as chemokines induction and integrin regulation and activation both for recruiting immune-suppressive accessory immune cells in the BM niche and for their own migration and survival. Therefore, the choice of such specific axes for engineering CAR T-cells may not only improve their migration into the leukemic niche but also target critical signals for leukemia own maintenance and immune evasion.

N. 1 linked to research project: *"iPSC modeling of Friedreich's ataxia and epigenetic reactivation of the Frataxin gene"*

Company: Istituto di Neuroscienze - Consiglio Nazionale delle Ricerche (CNR-IN)

Abstract:

Friedreich's ataxia (FA; OMIM 22930) is an autosomal-recessive neurodegenerative disorder mainly caused by silencing of the Frataxin (FXN) gene due to the expansion of a tract of GAA·TTC trinucleotide repeats (1). FXN is a nuclear gene that encodes a mitochondrial protein which plays several roles in iron metabolism and respiration, in particular in the biogenesis of iron–sulfur (Fe-S) clusters, leading to an impaired of Fe-S enzymes activity with altered cellular iron metabolism, reduction in energy production, and increased oxidative stress. We have generated high-quality iPSCs from two FA patients with either short or long GAA triplet expansions that correlate with milder and more severe disease symptoms, respectively.
In order to directly assess the impact of Frataxin gene silencing in different neuronal cell types, we will differentiate patient and isogenic control iPSCs into DRGO sensory neurons, retinal ganglion cells (RGCs) and cerebral cortical neurons to investigate and compare their cellular and molecular dysfunctions. iPSC- derived sensory neurons, RGCs and cortical neurons will follow the same functional analysis in order to evaluate their relative vulnerability to the pathological effects induced by Frataxin silencing. It is expected that sensory neurons will be more susceptible respect to cortical neurons, while RGC behavior remains to be fully explored. Mitochondrial morphology and functionality, oxidative stress and overall viability will be assessed in neuronal culture Subsequently, mitochondria trafficking along the axonal projections will be recorded by chronic time-lapse imaging on neurons plated on microfluidic devices. Altogether, this research program will contribute in revealing the pathological effects caused by Frataxin silencing in FA and revealing the molecular basis of the different susceptibility to the disease effects of different neuronal cell types.
Next, we will employ the Cas9-VPR and the Cas9-TET1 activator systems to stimulate the expression of the silenced Frataxin gene in patient cells. Initially, sgRNAs will be designed and validated on the Frataxin promotor region or more spread within proximal intron 1 for the Cas9-VPR and Cas9-TET1 experiments, respectively. Next, efficiency of Frataxin gene upregulation will be assessed in two lines of patient primary fibroblasts. The most performing sgRNAs will be employed with Cas9 effectors on iPSC-derived sensory neurons, RGCs and cortical neurons. The sgRNA which will sustain significant Frataxin gene upregulation in neurons will be further characterized for off-target activity by profiling the expression of candidate off targets or by unbiased RNA-seq transcriptome analysis. For the Cas9-VPR in particular, its expression will be silenced in cells and Frataxin upregulation will be tested to assess whether its effects are irreversible or not. Moreover, for the Cas9-TET1, bisulfite sequencing will be executed to determine the efficiency of DNA methylation and its correlation with Frataxin gene upregulation. This study will contribute to the development of a new approach to rescue the expression of the endogenous gene, and thus, avoiding detrimental effects associated with overexpression of the exogenous viral gene.

N. 1 funded by Department of Medicine and Surgery linked to research project:"*Establishment of a human stem-cell based microglia platform for the molecular investigation of brain development and disease***"**

Abstract

Microglia are increasingly implicated in brain development and pathology but the molecular underpinnings have not yet emerged. The project aims at the generation of a human platform of microglia-like cells derived from human pluripotent stem cells (hPSCs) and its use for the molecular characterization of microglia functions in neurodevelopment and disease. The project will rely on the differentiation of hPSCs carrying disease-associated gene alterations towards a microglia identity in 2D and 3D culture settings and their molecular and functional characterization through transcriptional profiling, immune assays and organoid-based phenotypic analysis. The results of this work will shed light on the mechanisms of microglia dysfunction in neurodevelopmental disorders, thus potentially provide novel therapeutic strategies.

PhD Executive Positions

N. 2 linked to research project:

1) *"Development of a cloud-based software tool for the genetic identification of circulating fetal trophoblasts isolated from a digital cell sorter"*
2) *"Detection of micro-imbalances and point mutations in single fetal trophoblasts isolated from maternal blood for prenatal diagnosis"*

Company: Menarini Biomarkers Singapore PTE, LDT

Abstract

Project 1
Development of a cloud-based software tool for the genetic identification of circulating fetal trophoblasts isolated from a digital cell sorter.

The discovery of the existence and the feasible isolation of circulating fetal trophoblasts from maternal blood represented a fundamental step for the implementation of new and promising technologies aimed at replacing invasive procedures for prenatal diagnosis. The recovery of a single intact cell enables the analysis of the whole fetal genome, breaking down the intrinsic limits of resolution and reliability due to the fragmentation of the genetic material and the low fetal fraction, typical of circulating cell-free DNA.
For some years our company has been working on the development of a highly processive workflow for the isolation of fetal trophoblasts from maternal blood, and their following genetic characterization through NGS-based techniques for copy-number profiling. The main goal is to obtain a diagnostic, automated and scalable protocol for potential future use in routine clinical practice.
However, cells isolated with this approach need to be confirmed as fetal cells, in order to avoid the characterization of a specifically-isolated maternal cells. Usually, fetal cells are identified using STR assays by comparing their profiles with maternal control. However, the necessary DNA amplification makes this approach less reliable, however requiring an additional assay with consequent dedicated time and resources. The reading of DNA sequences for copy-number profiling generally obtained in our workflow can be exploited as a source of information to be computationally used to discriminate fetal cells from maternal ones, thus achieving a reduction in terms of time and resources. Our company is already working on a prototype which has proven to have excellent performances in terms of discrimination.
This research project aims to improve this prototype with an optimization tailored on our workflow, using all the information that are available in the various steps, ranging from the cell sorter reports to the metrics obtained during copy-number profiling, implementing the most suitable and innovative methodologies for processing heterogeneous data (e.g. machine-learning). Moreover, this software tool needs to be optimized also at computational level, envisioning its potential use in cloud computing platforms and following implementation as a diagnostic tool.

Project 2
Detection of micro-imbalances and point mutations in single fetal trophoblasts isolated from maternal blood for prenatal diagnosis.

Currently, prenatal diagnosis is based on invasive procedures for the identification of possible pathological aneuploidies or micro-imbalances. These procedures (amniocentesis, CVS) are very accurate but present, although low, a certain risk of pregnancy termination. Therefore, in recent years there have been many efforts to develop non-invasive technologies, which remove the risk of fetal loss while also maintaining the accuracy required by diagnostic tests. The isolation of fetal DNA freely circulating in maternal plasma certainly represented a fundamental step in the path towards non-invasive procedures. However, some characteristics of this methodology limit its use to the identification of large alterations and, in any case, allow only a testing scope, where any positivity requires an invasive confirmation.
The discovery of trophoblastic cells circulating in maternal blood and of the possibility of their isolation opened

a promising new direction in this field of research, as the analysis of the entire intact genome of the fetus could overcome the limits of circulating cell-free DNA and allow a diagnostic-level result, even for small alterations.
For some years our company has been working hard in the isolation of fetal trophoblasts from maternal blood, and has developed a high-throughput workflow able to obtain genetic profiles of fetal cells starting from a maternal blood sample. This new technology has already been tested on a cohort of 380 pregnancies for which CVS or amniocentesis were performed, showing full agreement with the invasive results in terms of detected aneuploidies. In addition, a clinical performance evaluation study is currently ongoing with a cohort of 1,500 patients. However, aneuploidies are only the starting point, as our real goal is the analysis of microduplications / microdeletions and point mutations, i.e. those alterations in which placental mosaicism appears to have less impact and in which cell-free DNA has the most severe limits. Currently, the genetic profiles obtained with our approach are based on NGS sequencing and have shown a resolution of about 1.5Mb, a size sufficient to cover the main clinically-relevant alterations, however far from the resolution achievable with chromosomal microarrays obtained from cell pools. This limit is mainly due to the nature of the analyzed subject - single cells - whose DNA needs to be amplified in order to be subsequently sequenced. Therefore, the biases obtained from the amplification does not allow a uniform genome coverage, with a resulting significant impact on the resolution.
The research project that we would like to start aims to improve our current workflow with both laboratory and computational strategies able to overcome the current limitations of our technology, improving the resolution in the CNV detection and developing new approaches for the identification of known hereditary or *de-novo* pathological mutations. In this way, our technology will be able to achieve the detection capability necessary for a potential use in clinical practice.

N. 1 linked to research project: *"The Real-World-Evidence (RWE) methodology to study drugs for rare diseases: the Primary Biliary Cholangitis (PBC) case"*

Company: Intercept Italia S.r.l.

Abstract:

Primary biliary cholangitis (PBC) is a rare chronic cholestatic disease with autoimmune pathogenesis that can be progressive and lead to cirrhosis and liver failure.
Most patients with PBC do not complain any symptoms at diagnosis but 51% will become symptomatic within 5 years and 95% after 20 years[1]. Moreover, the predominance of females over males, initially in a ratio of 9: 1, today tends to be much lower: this has strengthened the importance of studying epidemiology, pathogenesis, complications, and therapy.
To date, the therapy approved for patients with PBC includes a first line with ursodeoxycholic acid (UDCA) and for non-responders it is carried out with a second line therapy with obeticholic acid (OCA)[2].
Currently, real-life data and real-world-evidence (RWE) studies are a crucial tool for continuing to study drugs and molecules, especially in the field of rare diseases, where disease incidence rates are low and there is still a great unmet need.
In PBC, real-life data play an important role in better understanding the efficacy and safety of OCA in clinical practice, especially in patients who are less represented or have been excluded from clinical trials.
In Italy, the experience with obeticholic acid was recently published on a sample of 311 patients with PBC and 191 subjects treated for at least 12 months with OCA[3] were analyzed. The study demonstrated that OCA therapy was globally effective, safe and well tolerated in patients treated for 12 months and that the real-life tool is characterized by a less rigid approach than the clinical trials and a more personalized treatment, which can be translated into a benefit for patients.
The objective of the project is to deepen the methodology of RWE studies as a useful tool for studying drugs for rare diseases. In particular, we want to use obeticholic acid as an example of real-life by evaluating it in a larger sample of patients and a longer follow-up duration, in order to obtain more robust data on long-term efficacy and safety.

N.1 linked to research project: *"Advanced genetic engineering of the hematopoiesis"*

Company: Dana-Farber Cancer Institute

Abstract:

To create a new treatment strategy for high-risk acute myeloid leukemia patients, we aim to modify specific surface molecules of normal hematopoietic stem cells used for allogeneic bone marrow transplantation in order to make them resistant to targeted therapies such as CAR-T cells. This would allow the administration of highly promising immune therapies without the risk of severe toxicity resulting from normal stem cell recognition and destruction. In particular, the PhD candidate will use the novel CRISPR- Cas gene editing technologies to modify the part of the target that is recognized by antibodies and CAR-T cells in order to prevent the immunotherapies from killing normal stem cells. Through this approach, only residual leukemia cells will be targeted by CAR-T cells, while transplanted human hematopoietic stem cells will be spared, while still retaining the full function of the modified gene. To test this approach, we have chosen to modify some genes that are fundamental for the survival of leukemia cells, thus reducing the risk of tumor escape by random mutation. Given the nature of the research project and the requirement of specific resources available only at INSTITUTION (transgenic mouse models, advanced analytical instruments and facilities, specific financial resources), it is foreseen that the experimental research activities will be conducted at the INSTITUTION location.